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# The Effect of Cortisol on Proliferative Properties of Flounder (*Paralychthys Olivaceus*) B Lymphocytes

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**Flounder B lymphocytes isolated from different tissues were studied in terms of cell proliferation, apoptosis and the effects of cortisol on these processes. B lymphocytes, isolated from the flounder head kidney and spleen, were characterized by higher proliferation and lower intracellular calcium ($Ca^{2+}_i$) response to Ig-crosslinking compared with peripheral blood B lymphocytes. Cortisol induced high levels of apoptosis (150% of control levels) in peripheral blood B lymphocytes, in combination with a stimulatory LPS signal. Head kidney and to a lesser extent spleen B lymphocytes, although less sensitive than their equivalent in peripheral blood, underwent cortisol-induced apoptosis irrespective of extra stimulation up to 142% of control levels. Also proliferation with and without LPS stimulation was suppressed by cortisol (compared to plasma values measured during stress conditions) that is effective in inducing a significant increase in apoptosis in all three populations of B-cells, suggesting that cortisol may be important for immunoregulation in both stressed and non-stressed conditions. This implies possible severe impact of stress on lymphocyte development and activity. Different sensitivity of B-cells to the corticosteroid, with respect to developmental stage and activity, may prevent excessive and long lasting depletion of B-lymphocytes.**

Bi-directional interactions of immune and endocrine functions are now recognized to be very important in the regulatory network, ensuring homeostasis during both stress and non-stressful conditions (Weyts et al., 1999). Given the need to develop disease control procedures in aquaculture, it is of great importance to reveal the mechanism of the neuroendocrine-immune system interactions in fish. Stressor-induced immunomodulation has mainly been attributed to cortisol (Ellis, 1981; Barton et al., 1991), the major corticosteroid in many fish, which is produced by cells in the interrenal tissue. Cortisol secretion is under endocrine control from the pituitary and the main mediators are ACTH and αMSH, which are enhanced during acute and chronic stress (Wendelaar Bonga, 1997).

Treatment of fish with cortisol resulted in reduction of: (1) leukocyte proliferation (Ellsaesser and Clem, 1987; Le Morvan-Rocher et al., 1995; Espelid et al., 1996); (2) numbers of antibody producing cells (Carlson et al., 1993; Mazur & Iwama, 1993); (3) levels of virus-neutralizing antibodies (Wechsler et al., 1986) and (4) circulating numbers of lymphocytes (Ellsaesser & Clem, 1987; Espelid et al., 1996).

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The mechanism of cortisol-induced immunosuppression has been the subject of several studies. The literature concerning the impact of stressors and corticosteroids on activity of phagocytic cells is not consistent, probably due to the difference in species, stress-protocols and assay systems (Weyts et al., 1999). In vitro studies have revealed that mainly B-lymphocytes appear to be directly affected by cortisol, manifested by reduced levels of proliferation (Grimm, 1985; Tripp et al., 1987; Espelid et al., 1996) and reduced antibody production (Tripp et al., 1987). Moreover, it has been shown that carp peripheral lymphocytes and head kidney neutrophilic granulocytes possess high affinity receptors for cortisol and that at least part of the impact of cortisol is mediated through these receptors and affects apoptosis in these cells (Weyts et al, 1998a). These effects of cortisol on cell viability are cell type specific and may be dependent on the differentiation and activation state of the leukocytes. Stimulated B-lymphocytes are especially sensitive and easily become apoptotic, whereas thrombocytes and cells of the T-lymphocyte fraction are insensitive to cortisol (Weyts et al., 1997, 1998b). In contrast, apoptosis of head kidney neutrophilic granulocytes was inhibited when cultured in the presence of cortisol (Weyts et al., 1998c). Clearly, as neutrophils, together with macrophages form a first line of defense against invasion by micro-organisms,

mobilization of these cells in conditions of stress may be important for survival.

In most studies the cortisol-induced changes in leukocyte function are associated to stressful events. However, considering the low concentration of cortisol that inhibits carp PBL proliferation in vitro (Weyts et al., 1997) one should keep in mind that endogenous cortisol may also be important in maintaining immunological homeostasis in fish, independent of a stress response.

The high sensitivity of peripheral B-lymphocytes, especially in the activated state, evoked our interest to study B-cell populations from the flounder haematopoietic head kidney (with high numbers of developing lymphocytes) and spleen as a secondary lymphoid organ, as well as from the peripheral circulation. Lymphocyte populations were characterized for surface immunoglobulin (sIg) expression, basal and lipopolysaccharide (LPS) stimulated proliferation and apoptosis, and effect of Ig-crosslinking on increases of intracellular calcium concentration. Subsequently the effect of cortisol was analyzed against non-stimulated and LPS-stimulated proliferation and apoptosis.

## Materials and Methods

*Animals*

Adult flounder, *Paralychthys olivaceus*, was obtained from a commercial flounder breeding farm. Fish was held at 23°C in recirculating, UV-treated water and fed pellet food, at a daily rate of 0.7% of their body weight. Animals were euthanized with 0.2 g/l tricaine methane sulphonate.

*Isolation of leukocytes*

Heparinized blood was obtained by puncture of the caudal vessel and mixed with an equal volume of RPMI 1640 medium (Gibco). After centrifugation (10 min at 100 ×g, followed by 5 min at 700×g) at 4°C with the brake disengaged, white cells in the buffy coat were collected and layered on 1.5 volumes of Lymphoprep (density= 1.077 g/ml, Gibco). Following subsequent centrifugation at 800×g for 25 min at 4°C, the leukocyte layer at the interface was collected, washed three times with RPMI and the final suspension was adjusted to $10^7$ cells/ml.

Head kidney and spleen tissue were dissected and cell suspensions were prepared by passing the tissue through a 50 µm nylon mesh. Cell suspensions were washed once before layering on a discontinuous Percoll gradient. Following centrifugation (800×g, 25 min), cells at the interface were collected and washed twice as above.

All cell suspensions were plated in 24 well culture plates at a density of $10^7$ cells/well and left to adhere for 1 h at 26°C and 5% $CO_2$, to remove neutrophilic granulocytes and monocytes/macrophages. Non-adherent cells were subsequently harvested by carefully pipetting off and suspended at a density of $10^7$ cells/ml.

*Leukocyte culture conditions*

Cells were seeded in 96-well plates ($10^6$ cells/well) and cultured overnight in RPMI containing $10^6$ IU/ml penicillin-G (Sigma, USA), 50 mg/l streptomycin sulphate (Serva, Germany) and 2.0 mmol/l L-glutamine. Cells received no stimulus, or were stimulated with lipopolysaccharide (LPS 100 ng/ml; *E. coli*: B5 LPS, Difco, Detroit, MI, USA) for 4 h at 27°C in 5% $CO_2$, followed by the addition of 0.5% pooled flounder serum (PFS, pooled serum from 20 adult flounder, containing 45 ng cortisol/ml, as determined by radioimmunoassay). Cortisol (36 ng/ml or $10^{-7}$ M) was added and cultures were maintained for 24 h and 48 h at 27°C in 5% $CO_2$. This cortisol concentration corresponds to half maximal free plasma cortisol concentrations in mildly stressed fish and induces substantial apoptosis in active PBL in vitro (Weyts et al., 1997).

*Measurement of leukocyte surface Ig-expression*

Leukocytes ($1.25\times10^6$/ml) were incubated for 30 min at 4°C with a monoclonal antibody (mAb) against flounder IgH chain, FIM 511 which was previously manufactured in our laboratory. Cells were washed and centrifuged for 7 min at 680×g at 4°C. They were resuspended in RPMI followed by incubation with fluorescein-isothiocyanate (FITC)-conjugated or rhodamine-conjugated rabbit-anti-mouse IgG (RAM-Ig) antibody (1:100; Dako A/S, Glostrup, Denmark) for 20 min at 0°C. After washing, $10^4$ cells were analyzed using a FACStar (Becton Dicknson, Mountain View, CA, USA) tuned at 488 nm using the DataMATE software (applied cytometry systems). Within the lymphocyte gate (Kouman-van Diepen et al., 1994), the percentage of cells stained with the antibodies was determined.

*Changes in intracelluar levels after Ig-crosslinking in fluo3-AM-loaded lymphocytes*

Measurement of changes in intracellular calcium was performed as established earlier (Verburg-van kernenade et al., 1998). Lymphocytes were loaded with fluorescent Ca indicator at a cell density of $10^7$/ml, at room temperature in the dark. Fluo3-AM, 4 µmol/l (Sigma, USA) was added from a 1 mM stock solution in dry dimethylsulfoxide (DMS). To improve the uptake-efficiency 6 µl/ml of Pluronic F-127 (Sigma, USA, 3% w/v in RPMI) was added. After 40 min, the loading solution was diluted 1:10 with RPMI, and after incubation for another 10 min, the cells were collected by centrifugation for 7 min at 700×g. The pellet was resuspended to obtain $1.25\times10^6$ cells/ml in RPMI. Cells were then incubated at 26°C before and during

$Ca^{2+}_i$ analysis.

Fluc-3 emission fluorescence in the cells was recorded with the flow cytometer at 530±30 nm. Baseline fluorescence was established at 5 min intervals (experimental samples were measured in paralled at 30s intervals). After cross-linking of sIg by addition of FIM 511 and RAM-Ig (Dakopat, Denmark) the fluorescence intensity was reassessed within a 10s time span and every 5 min thereafter.

*Measurement of lymphocyte proliferation*

Cultured cells were labelled with 185 KBq/ml, $^3$H-methyl thymidine (Amersham, UK) for 16 h. The content of each well was harvested with a squatron semi-automatic cell harvester (Lier, Norway). The filters with retained cells were dried for 1 h at 50°C and were counted in a Beckman LS 1701 scintillation counter using Beckman Ready Safe Scintillation Fluid. Measurements per fish were done in triplicate.

*Apoptosis measurements*

Following FIM 511 labelling as described above, cells were washed in RPMI supplemented with 1% BSA and 0.01% sodium azide. They were labelled with annexin V, conjugated to FITC (Boehringer, Mannheim, Germany), as described by the manufacturer. Annexin V has been shown to detect apoptosis in carp lymphocytes (weyts et al., 1998b). Green and red fluorescence intensities of cells within the lymphocyte gate were measured in the FACScan. In a parallel sample, propidium iodide (PI) exclusion was used to distinguish necrotic cells, which also expose phosphatidyl-serine, from apoptotic cells. Measurements per fish were performed in duplicate.

*Statistics*

Each tissue sample was collected from a different fish to ensure the independence of data. The difference between cells from the three populations was evaluated using one factor variance analysis (ANOVA) and revealed a statistical difference between these with regard to proliferation (t=1, NS, $P$=0) and basal apoptosis was not identical (t=0, NS, $P$=0.09). Therefore the effects of cortisol were evaluated comparing sensitivity of the leukocytes expressed as the percentage of the control. Differences in sensitivity among groups were assessed in 3 factor ANOVA with organ (3 levels-PBL, head kidney and spleen), stimulation (2 levels -HS and LPS) and time (2 levels-day 1 and 2) as factors, all orthogonal and fixed. If ANOVA was significant Student-Neuman-Keuls test was used to determine which means were significantly different. Prior to the ANOVA, Cochran's test was used to test for homogeneity of the variances. The results were considered to be statistically significant if $P<0.05$.

## Results

*B cell properties in leukocyte suspensions of head kidney, spleen and blood*

The percentages of B cells in the cell suspensions of non-adherent cells from head kidney, spleen and blood amounted to 29% (±3.2), 25% (±2.9) and 52% (±2.7) respectively.

Basal in vitro proliferation capacity and sensitivity to LPS stimulation is given in Fig. 1. Highest basal proliferation

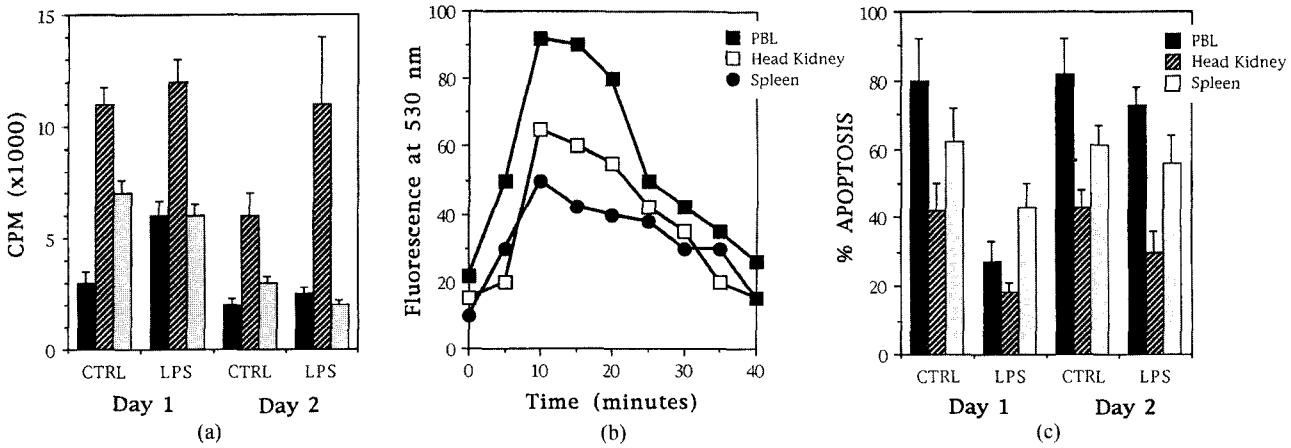


**Fig. 1.** (a) $^3$H Thymidine incorporation in vitro of non-adherent leukocytes from peripheral blood, head kidney and spleen in the absence and presence of 100 μg lipopolysaccharide (LPS)/ml after 24 and 48 h in culture. Bars represent the means of 3 fish ±S.E. (b) increase in average Fluo3 fluorescence (530 nm) measured at 6 min time intervals in non-adherent leukocytes of PBL, head kidney and spleen after Ig-crosslinking with WCI12-RAM. WCI12-RAM was added 10s before measurement at 6 min. (c) Percentage of apoptotic cells in non-adherent leukocyte populations from PBL, head kidney and spleen as measured by annexinV labelling in the absence and in the presence of 100 μg LPS measured by annexinV labelling in the absence and in the presence of 100 μg LPS/ml, measured initially (t=0) and after 24 and 48 h in culture. Bars represent the means of 9-10 fish ±S.E.

was found in cells of head kidney and spleen, whereas peripheral blood lymphocytes showed low proliferation. After 1 day of LPS stimulation, proliferation was increased significantly in head kidney and PBL. The highest absolute proliferation was again found in head kidney, with intermediate levels in spleen and PBL (Fig. 1a). Increases of proliferation were lower after 2 days of cell culture.

The B-lymphocytes in head kidney leukocyte suspensions reacted to sIg-crosslinking with the highest elevation of intracellular calcium levels as compared to spleen cells and PBL (Fig. 1b). To avoid differences in reaction due to differences in B cell numbers, suspensions with a maximum of 5% difference in B cell percentages were selected.

Basal apoptosis values directly after cell isolation were lowest in PBL (9.7%±2.2). After culture for 24 or 48 h, apoptosis levels increased and were highest in PBL. Head kidney B cells showed the lowest level of apoptosis after culture. After LPS (100 µg/ml) treatment for 24 and 48 h, levels of apoptosis significantly decreased. Decreases were most prominent in PBL and head kidney (Fig. 1c).

*Effect of cortisol on B cell proliferation in vitro*

Cortisol ($10^{-7}$ M) decreased the in vitro proliferation capacity of all cell populations (Fig. 2). After 1 day the effect on head kidney cells was small, reaching 27% reduction after 2 days of culture. Spleen cells were more affected, showing a 40% decrease of proliferation after 1 day and 80% after 2 days of culture. Peripheral blood leukocytes, which had a very low basic proliferation capacity in vitro, showed a decrease of 88% after 1 day (and 82% after 2 days) of culture. Combined cortisol and LPS treatment increased absolute proliferation in vitro, but resulted in a relatively higher cortisol induced reduction of proliferation as compared to non-stimulated cells. Again head kidney cells showed only limited sensitivity (maximum 42%).

*Effect of cortisol on in-vitro apoptosis*

Non-stimulated leukocyte fraction of PBL were least sensitive to cortisol treatment with respect to induction of apoptosis (Fig. 3). Levels amounted to an average of 5% and 10% above control level after 1 or 2 days of culture. Stimulation with LPS, however, brought about sensitivity to cortisol treatment. In this condition the level of apoptosis increased with an average of 50% after one day of culture and 15% after 2 days of culture (Fig. 3).

Whether tested in non-stimulated or LPS-stimulated conditions, head kidney leukocytes showed similar sensitivity to cortisol. Apoptosis levels in non-stimulated cells were 23% and 49% above control levels after 1 and 2 days of culture respectively. In LPS-stimulated conditions, average apoptosis levels of 25% and 42% above control values were registered after 1 and 2 days in culture.

Spleen cells showed sensitivity to cortisol-induced apoptosis after two days but not after one day of culture. Apoptosis reached 20% above the control level. After LPS treatment both the 1 day and the 2 days cell culture had undergone extra cortisol-induced apoptosis of 10%

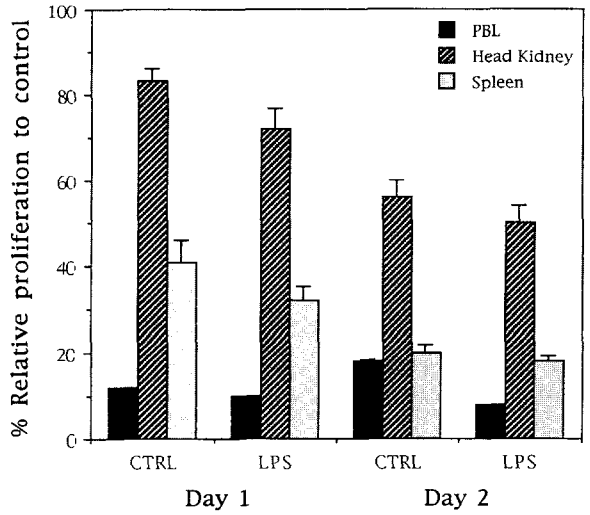


**Fig. 2.** Relative decrease of in vitro proliferation ($^3$H-thymidine incorporation) after 1 and 2 days of culture in the presence of $10^{-7}$ M cortisol in non-adherent leukocytes of peripheral blood, head kidney and spleen. NS are the cultures stimulated with 100 µg LPS/ml. Bars represent the means of three fish ±S.E. Cortisol inhibited proliferation in all organs ($P<0.01$) and all organs differed in their sensitivity to cortisol ($P<0.01$) at both times regardless of NS or LPS. NS and LPS were significantly different ($P<0.01$), time significantly affected proliferation ($P=0$).

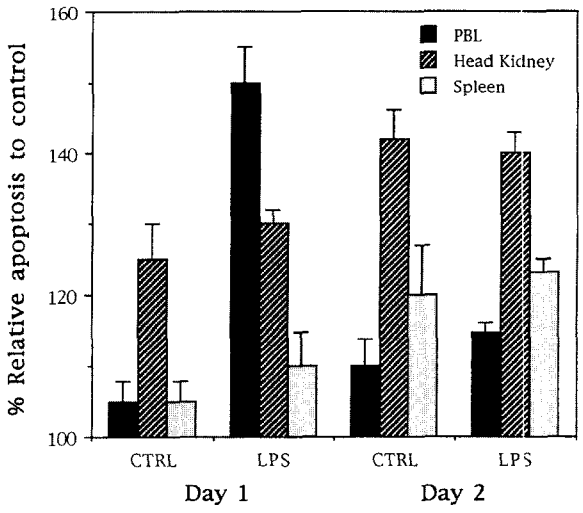


**Fig. 3.** Relative increase of apoptotic (AnnexinV positive) cells in non-adherent leukocyte populations of peripheral blood, head kidney and spleen after 1 and 2 days in vitro culture in the presence of $10^{-7}$ M cortisol. NS are the cultures of non-stimulated cells, LPS represent the cell cultures stimulated with 100 µg LPS/ml. Bars represent the means of 9-10 fish ±S.E. LPS different from NS at $P<0.01$. NS (days 1 and 2) all organs different at $P<0.01$. LPS day 1: both HK and SPL different from PBL at $P<0.01$ and from each other at $P<0.05$. LPS day 2: PBL and SPL different from HK at $P<0.01$ and from each other at $P<0.05$.

and 22%, respectively.

## Discussion

Most studies on B-lymphocyte function in fish are performed with PBL as these are readily obtained and consist of a relatively pure lymphocyte fraction. In terms of B cell function, however, one should realize that cell populations from different origins may differ according to developmental stage and state of activation. We, therefore, have studied B cell populations in head kidney, which represents a primary haematopoietic lymphoid organ in fish, spleen and peripheral leukocytes. The most striking difference between the three cell populations is the high level of basic proliferation in head kidney and spleen as compared to PBL. Indeed, as may be expected from haematopoietic cells, they show many cell divisions due to high percentages of developing cells. PBL that do not spontaneously proliferate can still be stimulated to proliferate by LPS. In regard to Ig-crosslinking, the PBL generated higher levels of intracellular calcium following cross-linking, indicating stronger cell activation. Both characteristics of PBL are consistent with their function in humoral defense mechanism.

Earlier studies demonstrated that cortisol is involved in immune regulatory mechanism of carp (Weyts et al. 1998a). Furthermore, B cells within the PBL are especially sensitive to cortisol, which evoked strong dose-dependent decrease in the level of proliferation and a massive onset of apoptosis. However, these cells only became sensitive following stimulation, confirming previous results (Weyts et al. 1998b). Under stressful state, implying high levels of endogenous cortisol, one would argue that the generation of an efficient humoral response would thus be severely affected. In the presence of immune stimuli, e.g. invading microorganisms, the circulating cell population would be relatively protected due to lower sensitivity to the corticosteroid. This is indicative of a function for cortisol in removal of activated lymphocytes following an immune response. These lymphocytes may be potentially harmful with respect to a greater chance of unwanted autoimmune reactions. Conditions of stress might cause this process to take place too rapidly. The high relative cortisol-induced reduction of proliferation in the non-stimulated PBL population seems to contradict this fact., This, however, may be explained by the fact that proliferation capacity of non-stimulated PBL is extremely low in absolute amount, and thus is most probably ascribed to a very small percentage of activated and thus cortisol-sensitive cells within this population.

The impact of the stressors on immune competence cannot solely be explained by effects of cortisol on circu ating lymphocytes; it will of course also depend on the effect of cortisol on the populations of developing B cells in the haematopoietic organs. With respect to proliferation capacity it may be concluded that in head kidney high cortisol levels of relatively short duration (<1 day) may have limited impact. However, longer cortisol treatment may be more harmful, as a 2 day treatment resulted in reduction of proliferation in both stimulated and non-stimulated conditions of approximately 40%. However, in comparison to results obtained with spleen cells and PBL, head kidney B cells seem to be most protected. In regard to apoptosis the results show that in contrast to non-stimulated PBL, head kidney B cells and to a lesser extent spleen B cells are sensitive to cortisol-induced apoptosis. This may be indicative of a role of cortisol in B-cell selection. It may also be assumed that this is explained by the fact that these are cells that have been stimulated to proliferate in vivo. Further stimulation with LPS in vitro hardly induced any extra effects. In mammals, immature T and B cells are easily induced into apoptosis by glucocorticosteroids, consistent with the role of the steroids in the selection process (Lenardo, 1997; Ashwell et al., 1996). For fish this issue has not yet been investigated.

Knowledge of the magnitude and the mechanism of stressor-induced immunomodulation in teleosts is important to improve culture facilities with respect to harmful effects of crowding, handling and transport. It is difficult to establish which stressor-induced effects are cortisol mediated. Moreover, considering the findings that physiologically low-stress concentrations of cortisol are effective in inducing increased apoptosis and inhibited proliferation, it may be concluded that cortisol-induced immunomodulation is an integral part of immune cell development and immunoregulation in fish, independent of stressors. In this respect it may be relevant that the head kidney in fish harbours both the interrenal steroidogenic cells and the haematopoietic cells, possibly enabling paracrine interactions between both cell types. For flounder we now conclude that the sensitivity of B cells to cortisol is dependent on the state of activation and/or development. This differential regulation in different immune organ may be important for physiological regulation of the total immune response in stress as well as non-stress circumstances.

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